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MECHANISM OF SPERM-OOCYTE INTERACTION DURING FERTILIZATION IN THE SURF CLAM *SPISULA SOLIDISSIMA*

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Abstract

Spisula oocytes were treated with Triton X-100 to investigate sperm receptor(s) on the surface membrane. The study shows that macromolecules with sperm receptor activity reside on the surface membrane of oocytes. The evidence is that: 1.) Triton X-100 treated oocytes fail to undergo GVBD on exposure to sperm; 2.) Triton X-100 treated oocytes incubated with oocyte extract are capable of sperm-induced reactivation; 3.) Incubation of sperm with oocyte extract interfered with the fertilizing capability of sperm; 4.) Incubation of oocyte extract with sperm eliminated its ability to reactivate Triton X-100 treated oocytes to undergo GVBD; 5.) Reactivation of Triton X-100 treated oocytes is species-specific, *i.e.*, *Spisula* oocyte extract was active, while *Arbacia* oocyte extract was inactive; 6.) the lectin that interacts with fucose blocked GVBD induced by sperm.

Introduction

Fertilization is a complex sequence of events that involves species-specific fusion of plasma membranes of the sperm and the egg. It is likely that the initial interactions between the sperm and the egg are mediated by surface macromolecules that facilitate species specificity and binding (Schmell, *et al.*, 1977). Although the morphological events involved in the sperm-egg fusion have been extensively studied (Franklin, 1965; Longo and Anderson, 1970; Giudice, 1973), little is known about the molecular aspects of this process. Studies in the sea urchin (Summers and Hylander, 1975) and mammals (Hartman and Hutchinson, 1974) have shown that sperm adhesion to the egg investing layer is a species-specific phenomenon. For many years it was assumed that the sea urchin jelly coat functioned as the sperm receptor during fertilization (Lillie, 1914). However, it has been shown by others (Tyler, 1948; Summers and Hylander, 1975) that interaction between the egg jelly coat and the sperm are not necessarily species-specific.

The purpose of the work reported here was to investigate whether there are sperm recognition macromolecules on the surface of the surf clam *Spisula solidissima* oocytes that are responsible for species specificity and binding during the fertilization process. The surf clam oocyte has a great advantage for study because it contains a large germinal vesicle with a membrane that breaks down readily on

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Abbreviations: Con A, *Concanavalia ensiformes* agglutinin; DBA, *Dolichos bifluorus* agglutinin; GVBD, germinal vesicle breakdown; PNA, peanut (*Arachius hypogaea*) agglutinin; RCA-1, *Ricinus comunis* agglutinin; SBA, soy bean (*Glycine max*) agglutinin; UEA, *Ulex europaius* agglutinin; WGA, wheat germ (*Triticum vulgaris*) agglutinin.

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fertilization (Schechter, 1941; Allen, 1953). Thus the breakdown of the nuclear membrane can be used to determine the occurrence of fertilization in oocytes.

To establish the presence of macromolecules responsible for recognition and binding, the following criteria need to be fulfilled: the sperm receptor macromolecules must be located on the surface of the oocyte, *i.e.*, either on the plasma membrane or on the vitelline layer, and be saturable; in addition, the interaction should be linked to biological activity (*i.e.*, GVBD) and species-specific; when isolated, the oocyte surface macromolecules should inhibit fertilization by binding to the sperm counterpart; oocytes denuded of the active macromolecules must fail to show fertilizability; addition of the removed oocyte surface macromolecules back to the denuded oocytes should restore fertilizability of these oocytes.

Materials and Methods

Collection of gametes

Spisula solidissima and *Arbacia punctulata* were obtained from the Animal Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. They were maintained at 14°C in tanks containing running natural sea water. Sex was determined and large numbers of gametes were obtained by the method outlined by Allen (1953). Briefly, the two valves of the shell were separated by cutting the adductor muscles and removing the visceral mass intact. The gonads were exposed and excised.

Oocytes: The excised ovary was dissected in 200 ml of filtered sea water and strained through cheesecloth into a large beaker containing 200 ml of fresh filtered sea water. The oocytes were allowed to sediment to the bottom of the beaker and the supernatant removed. This process was repeated three times. The oocytes could be kept at room temperature for 2 h without deterioration.

Spermatozoa: The excised testes were also minced in 200 ml of filtered sea water and filtered twice through cheesecloth prior to storage on ice. Alternatively, "dry" spermatozoa were obtained by the method outlined by Allen (1953). Testes were incised and kept on ice for 1 h. At the end of this period, a milky sperm fluid had oozed out of the testis. This fluid was collected with a syringe and maintained on ice until used.

Fertilization of Spisula oocytes in vitro: *standardization of the Germinal Vesicle Breakdown Assay (GVBD)*. Germinal vesicle breakdown was determined by the methods of Schechter (1941) and Allen (1953). Using various concentrations of the sperm suspension against a fixed number of oocytes in Petri dishes containing filtered sea water, a sperm/oocyte ratio was reached at which maximum fertilization as determined by GVBD occurred after 10–15 min depending on the condition of the oocytes (Fig. 1). Percent GVBD was determined by counting the number of oocytes in a total count of 200 oocytes. The dishes were set up in duplicate. The variation between duplicate dishes was ±4%. To each dish containing approximately 5,000 oocytes in filtered sea water predetermined concentrations of spermatozoa were added. GVBD was determined by phase contrast microscopy.

Treatment with Triton X-100: An experiment to determine the effect of Triton X-100 on the fertilizability of *Spisula solidissima* oocytes was performed. Fixed amounts of oocytes were treated with various concentrations of the detergent and percent GVBD determined (Fig. 2). For most of the experiments, about 5,000 oocytes in 1 ml of filtered sea water were used. The oocytes were treated with detergent, washed three times by serial transfer into fresh filtered sea water dishes, and the spermatozoa added to the last dish containing the oocytes in duplicate.

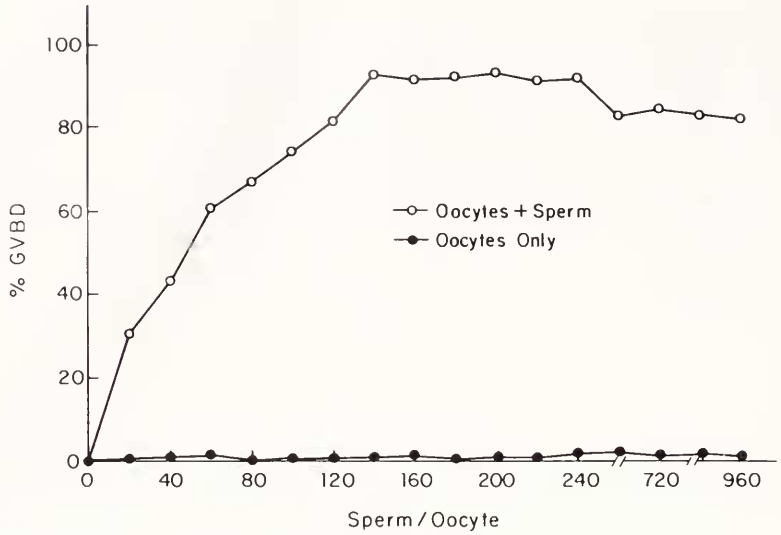

FIGURE 1. Induction of germinal vesicle breakdown (GVBD) at various concentrations of sperm per oocyte. GVBD observed 30 min after insemination at 25°C. (n = 4).

Preparation of oocyte extract: To investigate further what had happened to the *Spisula* oocytes after treatment with Triton X-100, a supernatant obtained from the detergent-treated oocytes was used to restore fertilizability to these oocytes. For this purpose the supernatant designated "oocyte extract" was treated as outlined in Figure 3. The supernatant was treated with several batches of SM-2 beads

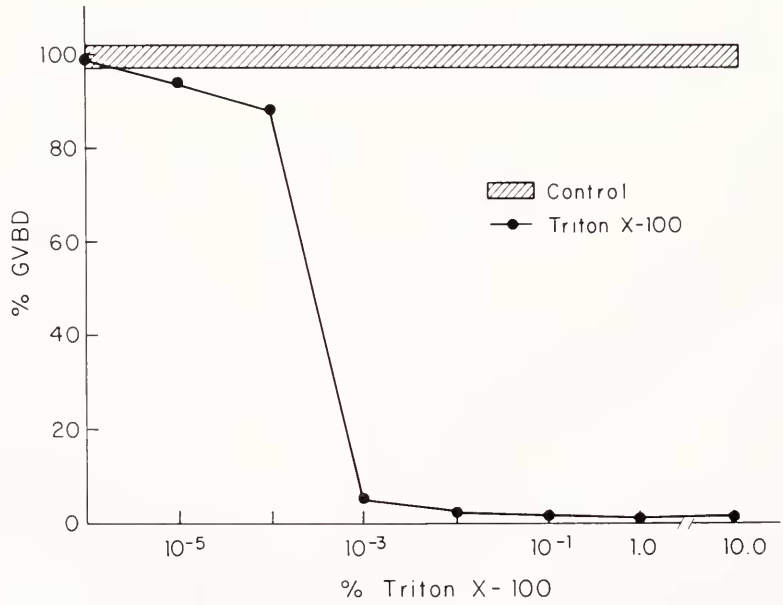

FIGURE 2. Effect of varying concentrations of Triton X-100 on GVBD in *Spisula* oocytes *in vitro*.

(Bio-Rad, Richmond, California) by the method of Holloway (1973) to remove Triton X-100. After concentrating with polyethylene glycol (PEG 20,000, Fisher Instruments) the total protein in the extract was estimated (Lowry *et al.*, 1951) and subsequently used in reconstitution experiments.

Viability of oocytes after treatment with Triton X-100: To determine whether the detergent at the concentrations used was cytotoxic to the oocytes, viability of the oocytes was established by observing the number of cells stained on exposure to 0.8% Trypan blue after serial transfer washing (Fig. 4).

Reconstitution experiments

(a) *GVBD in detergent-treated oocytes after the addition of the processed "receptor extract."* To determine what had occurred to the oocyte surface, the detergent-treated and washed oocytes designated as "stripped" oocytes (Fig. 3) were incubated with the oocyte extract containing different amounts of protein, for 60 min, washed by serial transfer and the GVBD determined.

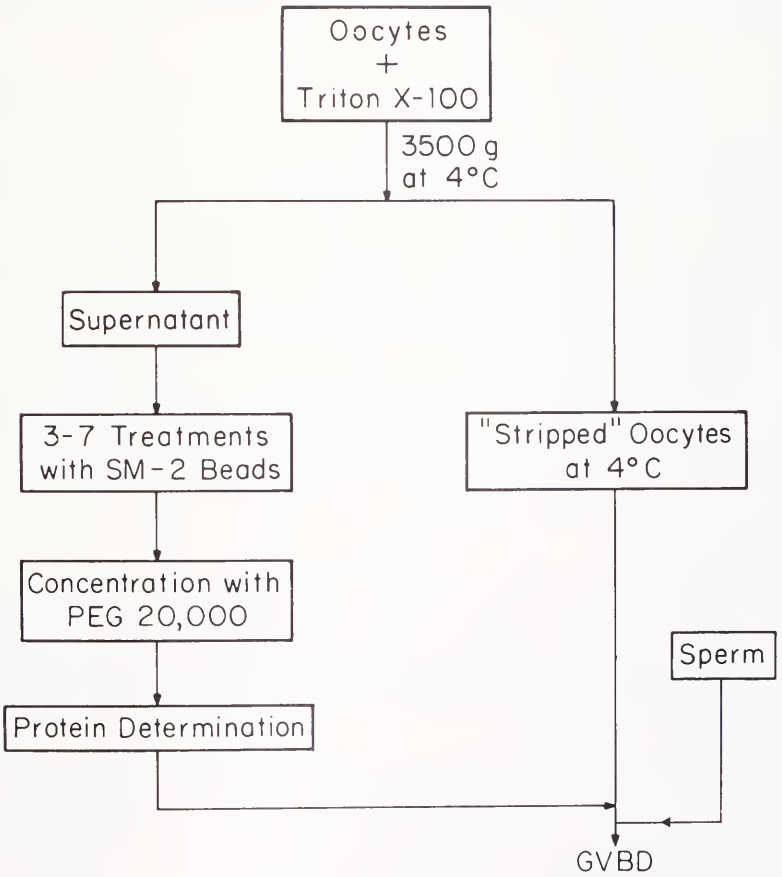

FIGURE 3. Outline of the procedures used for the extraction and processing of *Spisula* oocyte "receptor extract" used in the reconstitution experiments. PEG—polyethylene glycol.

(b) *Species-specificity of oocyte receptor extract in the restoration of GVBD.* To test the hypothesis that a molecular species removed from the oocyte surface by detergent was involved in species-specific recognition and binding, a soluble receptor extract was prepared from the sea urchin *Arbacia punctulata* oocytes using a modification of the technique of Schmell and colleagues (1977). *A. punctulata* oocytes were obtained by the electrical method of Harvey (1956). The oocytes were incubated at room temperature for 1 h and washed three times with freshly filtered sea water. They were lysed by suspension in distilled water by centrifugation for 10 min at 1000 × *g*. This process was repeated. The resulting pellet was resuspended in freshly filtered sea water and homogenized by 10 gentle strokes in a ground glass homogenizer. The resulting homogenate was dialysed overnight against freshly filtered sea water at 4°C and a soluble oocyte receptor extract obtained by centrifugation of the retentate for 30 min at 12,000 × *g*. Both the supernatant and the pellet were stored at −60°C until used. To determine whether the supernatant or the pellet contained "receptor activity" both were tested for ability to restore fertilizability to trypsin-treated *Arbacia punctulata* oocytes (Schmell *et al.*, 1977). Both the supernatant and the pellet were found to contain a component that could restore fertilizability to the reconstituted *Arbacia* oocytes. Only the supernatant was used in reconstitution experiments with *Spisula.*

(c) *Effect of the* Spisula *oocyte receptor extract on the capability of* Spisula *sperm to induce GVBD in normal oocytes.* The reduction of GVBD in oocytes after treatment with detergent not only suggests the existence of surface molecules on the oocyte, but also the presence of complementary macromolecules on the sperm surface. If *Spisula* spermatozoa contain specific surface molecules that combine with complementary molecules on the oocyte surface in a key-and-lock fashion, then the treatment of normal spermatozoa with the oocyte receptor extract should block GVBD. Before testing this hypothesis, the capability of the oocyte receptor extract to bind to normal sperm was determined. Spermatozoa (5×10^3) in 1 ml of filtered sea water were incubated for 30 min with the oocyte extract, centrifuged twice for 10 min at 12,000 × *g*, and the supernatants used for the restoration of GVBD in detergent-treated oocytes.

(d) *Treatment of oocytes with lectins*: To determine what type of sugars are involved in *Spisula* oocyte-sperm interaction, oocytes were incubated with 100 μg/ml of various lectins for 30 min, washed and percent GVBD determined following insemination.

Results

Germinal vesicle breakdown assay: An asymptotic curve was obtained when experiments were done to determine optimal ratio of sperm/oocyte concentrations at which maximum GVBD would occur. One representative experiment is depicted in Figure 1. The ratio of sperm to oocyte increased until there were about 140 spermatozoa per oocyte. Higher sperm/oocyte ratios did not significantly improve percent GVBD.

Effect of Triton X-100 on germinal vesicle breakdown: Figure 2 shows that Triton X-100 concentrations greater than 0.0001% inhibited GVBD in *Spisula* oocytes.

Viability: Viability of the detergent-treated oocytes was unaffected by concentrations of Triton X-100 lower than 0.0001% (Fig. 4).

Reconstitution experiments: Reconstitution experiments indicated that incubation of "stripped oocytes" with the oocyte extract restores GVBD to some oocytes

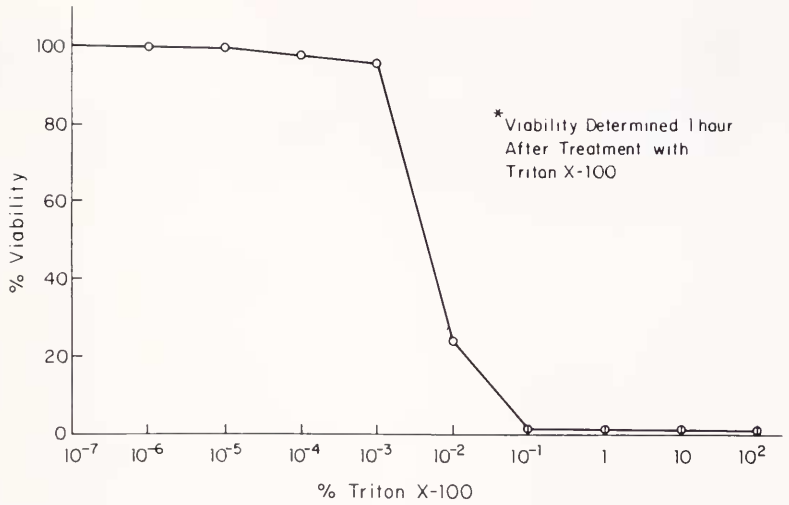

FIGURE 4. Viability of *Spisula* oocytes treated with varying concentrations of Triton X-100. Viability determined 60 min after Triton X-100 treatment by observing uptake of trypan blue. T = 22–24°C.

after 60 min (Table I), and that the heat-treated extract failed to restore GVBD capability to Triton X-100 treated oocytes while untreated extract restored GVBD to 21.6% (Fig. 5). Incubation of the "stripped oocytes" with the oocyte extract for longer than 60 min did not significantly improve percent GVBD. The percentage of reconstituted oocytes that underwent GVBD ranged from 15.4 ± 2.6 to 23.7 ± 3.9 (6 experiments) (Mean ± S.D).

Species specificity: Table II shows that only the *Spisula* oocyte membrane extract restored GVBD capacity to "stripped" *Spisula* oocytes. An *Arbacia* extract failed to restore GVBD capacity to "stripped" *Spisula* oocytes, suggesting that the ability of *Spisula* oocyte receptor extract to restore GVBD capacity to "stripped" *Spisula* oocytes may be species-specific.

Complementary sperm receptor: Pre-treatment of the *Spisula* oocyte extract with *Spisula* spermatozoa to absorb the sperm interacting proteins abolished the ability of the extract to restore GVBD capacity to stripped oocytes but had no effect on sperm motility (Table III). Triton X-100 treatment might have induced acrosome reaction in the sperm and reduced the fertilizing capacity. In addition,

TABLE I

Influence of oocyte extract containing sperm receptor on germinal vesicle breakdown in Triton X-100-treated Spisula *oocytes induced with sperm. GVBD in untreated oocytes, 95–98%.*

Incubation period (min.)	GVBD (%)	
	With extract (0.4 mg protein/ml)	No extract
15	2	0
30	4.3	0
60	17.8	1
120	16.9	1

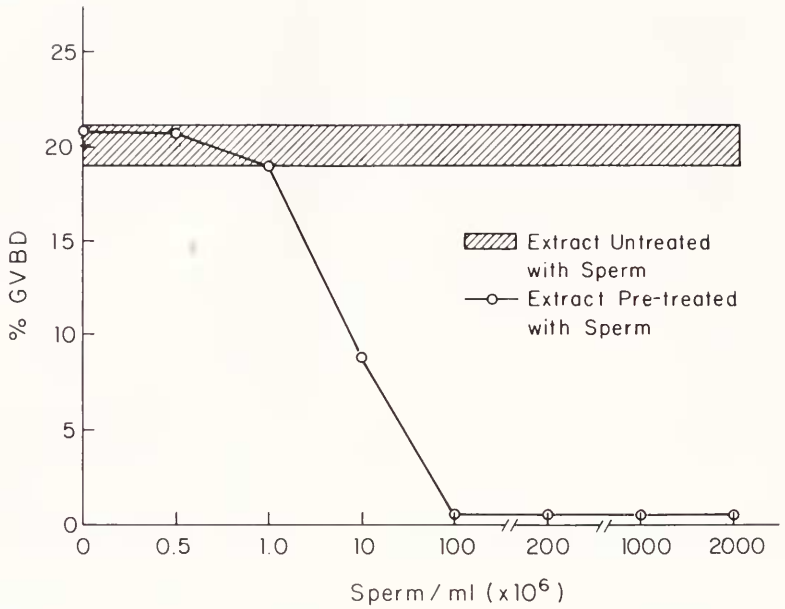

FIGURE 5. Capacity of oocyte extract absorbed with *Spisula* sperms to induce GVBD in "stripped oocytes." Varying concentrations of *Spisula* sperm were added to each ml of oocyte extract at 22°C for 30 min. The sperm were separated by centrifugation at 17,000 *g* for 10 min. The supernatant was tested for its capacity to restore the ability to undergo GVBD in Triton X-treated oocyte upon insemination. (n = 3).

the spermatozoa that had been pre-treated with the extract were unable to induce GVBD in normal oocytes (Table III and Fig. 5).

Inhibition of GVBD by lectins: Table IV shows that of the lectins tested for their involvement in sperm-oocyte interaction, only UEA with specificity for α-fucose was found to be directly involved. Treatment of the oocytes with 100 μg/ml of UEA, prior to insemination, reduced GVBD by 50%.

DISCUSSION

The rationale for conducting a study to determine the effect of Triton X-100 on *Spisula* oocytes was based on the premise that the oocyte surface contained

TABLE II

Influence of heterospecific oocyte extract containing sperm receptor on GVBD in Triton X-100-treated Spisula *oocytes.*

Incubation period (min.)	GVBD (%)	
	Sea urchin extract (0.32 mg protein/ml)	*Spisula* extract (0.4 mg protein/ml)
15	0	6.0
30	0	9.0
60	0	13.6
120	0	13.9

TABLE III

Influence of oocyte extract containing "sperm receptor" on sperm motility and fertilizability of Spisula *oocytes. Sperm preincubated with oocyte extract for 30 min and washed before use.*

	Sperm motility (%)		GVBD (%)	
Experiment no.	With extract (0.32 mg protein/ml)	No extract	With extract (0.42 mg protein/ml)	No extract
1	62	71	12	76
2	58	64	7	71
3	74	69	9	81

sperm-recognizing macromolecules and that these macromolecules could be extracted by the detergent without destroying their biological activity (Maddy and Dunn, 1976). Removal of macromolecules such as insulin receptor (Cuatrecasas, 1972, 1974), cholinergic receptor (Meunier, *et al.*, 1974) in biologically active form has been achieved. The present results show that treatment of oocytes with Triton X-100 removed a molecule or set of molecules necessary for recognition and binding. This is further supported by the sperm-oocyte ratios necessary for GVBD to occur as depicted in Figure 1. The asymptotic part of the curve implies saturation of the oocyte surface with sperm, *i.e.*, the oocyte surface has only a fixed number of sites to which spermatozoa can bind.

It is not clear whether the sperm binding sites or macromolecules on the *Spisula* oocyte are located primarily on the vitelline layer or on the oolemma which is projected into branched microvilli extending into the fibrous vitelline layer. A narrow perivitelline space extends from the oolemma to the lower edge of the vitelline layer. Figure 6 is a diagrammatic representation of an unfertilized *Spisula* oocyte drawn from an electron micrograph (Longo and Anderson, 1970). The electron micrograph shows that some of the microvilli extend to the surface of the vitelline layer. The exact localization of the receptor(s) was not determined although studies in the sea urchin (Aketa, 1975) suggest that they are located only on the vitelline layer.

Further evidence that specific macromolecules on the oocyte surface are involved in sperm-oocyte interaction was provided by our reconstitution experiments. About 20% of the oocytes were viable and able to undergo GVBD after re-incubation with

TABLE IV

Effect of lectins on GVBD in Spisula *oocytes. Oocytes pre-incubated with lectins (100 μg/ml) for 30 min and washed before use.*

Lectin competitor	Sugar specificity	GVBD (%)
Con A	α-D-Mannose, α-D-Glucose	98.0
PNA	D-Gal-β(1,3)-GalNAc	100.0
DBA	N-Acetyl-α-D-Galactosamine	95.0
RCA-I	β-D-Galactose	100.0
SBA	N-Acetyl-α-D-Galactosamine D-Galactose	99.0
WGA	N-Acetyl-β-(1,4)-D-Glucosamine 2(sialic acid)	100.0
UEA	α-L-Fucose	47.4

Controls ranged from 95.5–100%; 200 sperm/oocyte in each culture dish.
Values are the mean of 4 separate determinations.

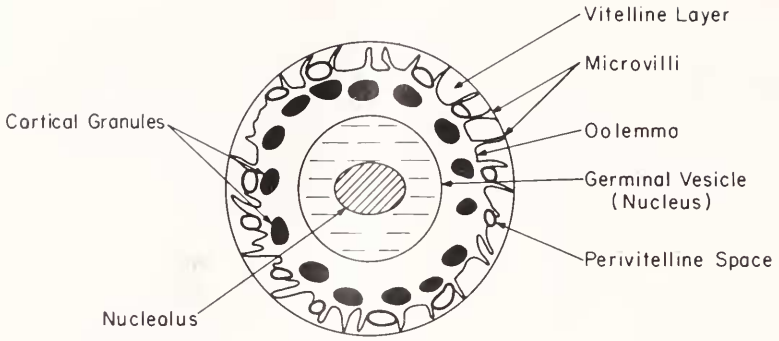

FIGURE 6. Diagrammatic illustration of an unfertilized *Spisula* oocyte. Drawing based on electron micrograph by Longo and Anderson (1970) showing possible sperm receptor sites on the oolemma or vitelline layer.

oocyte extract (Table I), suggesting that only a few oocytes can be reactivated to undergo GVBD. Perhaps the detached macromolecules underwent conformational changes and lost their capacity to reattach to their original or other corresponding sites, or the vitelline membrane was drastically altered. Membranes of a number of oocytes treated with 0.0001% Triton X appeared disrupted when viewed under the scanning electron microscope, indicating that these cells probably sustained irreparable injury.

One criterion for a molecule or set of molecules to act as a sperm receptor is species-specificity. Indeed, although human sperm will bind to hamster eggs *in vitro*, they will not fertilize these eggs (Gwatkin, 1976). It has been extensively shown that cross fertilization, though not absolute, fails to occur readily between different species of sea urchin (Kato and Sugiyama, 1978; Tyler and Tyler, 1966). The present finding that *Arbacia* egg receptor extract did not promote GVBD in detergent-treated *Spisula* oocytes supports the thesis that recognition and binding were species-specific processes.

Several workers have shown that in sea urchin (Aketa, 1973; Lallier, 1972) and in mammals (Gwatkin, 1976) Con A inhibits fertilization of eggs and that a receptor obtained from the egg surface binds to Con A (Schmell *et al.*, 1977). This finding suggests that the oocyte surface receptor contains either α-glucosyl or α-mannosyl units. When *Spisula* oocytes were treated with 100 μg/ml of various lectins, only UEA affected the capacity of the oocytes to undergo GVBD. The other lectins had no significant effect (Table IV). This indicates that α-fucose is involved in the sperm-oocyte interaction and that the fucose-containing molecule is located on the sperm. The involvement of fucose residues in sperm-egg interaction of *Ciona intestinalis* has been reported (Rosati *et al.*, 1978; Rosati and DeSantis, 1980).

The nature of the receptive molecule(s) on the egg suface is beginning to be explored. Early results in the sea urchin (Hagstrom, 1956) suggested that the receptor was altered by digestion with trypsin-like proteases. This was confirmed by Vacquier *et al.* (1972) and Aketa *et al.* (1968) who showed that digestion of the sea urchin egg surface with trypsin decreased fertilizability. A putative receptor protein was isolated from the surface of the sea urchin *Hemicentrotus* which when incorporated into air bubbles led to the binding of sperm to the bubbles (Aketa *et al.*, 1968). Although this material was not purified to homogeneity, it appeared to be a 2.3 S glycoprotein. Antiserum directed against this component blocked

fertilization (Aketa and Onitake, 1969) and sperm treated with the component lost their fertilizing ability but not their motility (Aketa, 1973). Furthermore, sea urchin sperm treated with the component did not agglutinate or undergo the acrosome reaction. Thus the factor seems to bind sperm before the acrosome reaction occurs. This makes it less likely to be the component involved in species-specific binding although the antisera directed against it blocks fertilization in a species-specific fashion. This material seems to have some, but not all, of the binding characteristics expected from physiological, morphological and from *in vitro* and *in vivo* studies of sperm-egg interaction.

A similar approach was used to detect a component from hamster zona pellucida that was suggested to be involved in the binding reaction (Hartman *et al.*, 1972; Gwatkin and Williams, 1977). The component showed some cross-reactivity in the mouse system. More recently, a glycoprotein in mouse egg zonae pellucidae with receptor activity for sperm has been identified. The glycoprotein from zonae pellucidae of unfertilized eggs called ZP3 reduced the binding of sperm to eggs *in vitro* (Bleil and Wassarman, 1980).

Another factor suspected of being a sperm receptor of the egg has been obtained from a membrane protein preparation of *Arbacia* eggs that inhibited fertilization of *Arbacia* but not *S. purpuratus* (Schmell *et al.*, 1977). Trypsin treatment of the factor destroyed its blocking potential. The soluble form of the factor binds to sperm and to Con A. The *Spisula* oocyte membrane material, however, does not bind to Con A. These observations suggest that the oocyte surface factors of *Spisula* and *Arbacia* contain different carbohydrate moieties.

An approach similar to the one employed in our work to obtain a sperm receptor was reported (Vacquier and May, 1977; Glabe and Vacquier, 1978). An attempt was made to isolate the vitelline layer by Triton X-100 and EDTA treatment of eggs. These vitelline layer preparations contained microvillous projections similar to those seen on the egg and sperm adhered to them only on the external surface. When the vitelline layer preparations were iodinated and examined by polyacrylamide gel electrophoresis, two high molecular weight components were seen. A high molecular weight, trypsin-sensitive glycoprotein obtained from this preparation (Glabe and Vacquier, 1978) has specific affinity for binding a particular component of a Triton X-100 extract of sperm. This would agree with our finding in *Spisula* that a complementary receptor molecule to the oocyte receptor exists on the sperm surface.

Acknowledgments

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